

Citation for published version:

Chakraborty, N, Sharma, P, Kanyuka, K, Pathak, RR, Choudhury, D, Hooley, R & Raghuram, N 2015, 'G-protein-subunit (GPA1) regulates stress, nitrate and phosphate response, flavonoid biosynthesis, fruit/seed development and substantially shares GCR1 regulation in *A. thaliana*', *Plant Molecular Biology*, vol. 89, no. 6, pp. 559-576. <https://doi.org/10.1007/s11103-015-0374-2>

DOI:

[10.1007/s11103-015-0374-2](https://doi.org/10.1007/s11103-015-0374-2)

Publication date:

2015

Document Version

Peer reviewed version

[Link to publication](#)

The final publication is available at Springer via: <http://dx.doi.org/10.1007/s11103-015-0374-2>

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G-protein α -subunit (GPA1) regulates stress, nitrate and phosphate response, flavonoid biosynthesis, fruit/seed development and substantially shares GCR1 regulation in *A. thaliana*

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Abstract

Heterotrimeric G-proteins are implicated in several plant processes, but the mechanisms of signal-response coupling and the roles of G-protein coupled receptors in general and GCR1 in particular, remain poorly understood. We isolated a knock-out mutant of the *Arabidopsis* G-protein α subunit (*gpa1-5*) and analysed its transcriptome to understand the genomewide role of GPA1 and compared it with that of our similar analysis of a GCR1 mutant (Chakraborty et al. 2015 (PLoS ONE 10(2):e0117819; 2015). We found 394 GPA1-regulated genes spanning 79 biological processes, including biotic and abiotic stresses, development, flavonoid biosynthesis, transcription factors, transporters and nitrate/phosphate responses. Many of them are either unknown or unclaimed explicitly in other published *gpa1* mutant transcriptome analyses. A comparison of all known GPA1-regulated genes (including the above 394) with 350 GCR1-regulated genes revealed 114 common genes. This can be best explained by GCR1–GPA1 coupling, or by convergence of their independent signaling pathways. Though the common genes in our GPA1 and GCR1 mutant datasets constitute only 26 % of the GPA1-regulated and 30 % of the GCR1-responsive genes, they belong to nearly half of all the processes affected in both the mutants. Thus, GCR1 and GPA1 regulate not only some common genes, but also different genes belonging to the same processes to achieve similar outcomes. Overall, we validate some known and report many hitherto unknown roles of GPA1 in plants, including agronomically important ones such as biotic stress and nutrient response, and also provide compelling genetic evidence to revisit the role of GCR1 in G-protein signalling.

Keywords

Arabidopsis thaliana

G-protein

G α

GPA1

GPCR

GCR1

Development
Flavonoid
Nutrient
Stress
Transcriptome
Functional genomics
Microarray

Abbreviations

GPA1 G-protein α subunit
GCR1 G-protein coupled receptor
GEO Gene expression omnibus
GO Gene ontology
SEA Singular enrichment analysis

Data Access: GEO accession number GSE 40217 (GSM 988507, GSM 988508, GSM988505 and GSM988506).

Electronic supplementary material

The online version of this article (doi:10.1007/s11103-015-0374-2) contains supplementary material, which is available to authorized users.

Introduction

Plants are known to have heterotrimeric G-proteins for over two decades (Temple and Jones 2007; Urano et al. 2013), but their organism-wide roles are yet to be understood. Only one or few isoforms of each of their subunits have been found in most plants so far, unlike in animals, which have whole families comprising of over 23 G α subunits, 6 G β subunits and 12 G γ subunits.

Arabidopsis is reported to have one α subunit (GPA1) (Ma et al. 1990), one β subunit (AGB1) (Weiss et al. 1994) and three γ subunits (AGG1-3) (Chakravorty et al. 2011; Mason and Botella 2000, 2001) so far. In soybean (*Glycine max*), recent studies revealed as many as 4 G α and G β subunits along with 10 G γ subunits, which correspond to a total of 160 possible heterotrimeric combinations, the highest reported in any plant for now (Bisht et al. 2011; Koepp et al. 2011). There is no evidence for the existence of multiple G α genes

in any other plant so far, but it cannot be ruled out either. Other unconventional G proteins, such as the extra-large G-proteins (Ding et al. 2008; Lee and Assmann 1999) and GPCR type G proteins (GTG1, GTG2) have been also been described (Pandey et al. 2009). Recently, it has been shown that extra-large G-proteins (XLGs) partner with G $\beta\gamma$ to mediate plant immunity (Maruta et al. 2015) and sometimes actually compete with G α to mediate several responses including tunicamycin, salt, and glucose hypersensitivity (Chakravorty et al. 2015).

The α subunit of plant heterotrimeric G-proteins has been implicated in various roles such as hypocotyl elongation, hook angle, rosette diameter, leaf shape (Ullah et al. 2001), ABA inhibition of stomatal opening (Wang et al. 2001), positive regulation of stomatal density (Zhang et al. 2008), pollen tube development (Wu et al. 2007), auxin mediated cell division, lateral root proliferation (Ullah et al. 2003), plant height (Fujisawa et al. 1999; Ueguchi-Tanaka et al. 2000), grain size (Oki et al. 2005), sphingolipid signaling (Coursol et al. 2003), resistance to pathogens (Komatsu et al. 2004; Suharsono 2002), sugar perception (Huang et al. 2006), light regulation of nitrate reductase gene expression (Ali et al. 2007; Raghuram et al. 1999) and blue light-induced production of phenylalanine (Warpeha et al. 2006, 2007). The other two G-protein subunits, β and γ , have also been implicated in several responses like cell division (Ullah et al. 2001), organ shape and size (Chen et al. 2006; Lease et al. 2001; Li et al. 2012), **gibberellin (GA)** biosynthesis (Chen et al. 2004; Ullah et al. 2003), control of sugar, **GA gibberellin** or brassinosteroid mediated inhibition of germination (Chen et al. 2004), control of guard cell ion channels and response to ABA (Chakravorty et al. 2011), resistance to necrotrophic pathogens, jasmonate signaling (Ishikawa 2009; Llorente et al. 2005; Trusov et al. 2006) and oxidative stress responses (Joo et al. 2005).

But the precise role of GPA1 in the regulation of any of the above processes has not been fully established and the molecular details of signal-response coupling remain to be elucidated. Taking advantage of functional genomics, attempts have been made recently to dissect the genome-wide role of G-protein subunits, GPA1 and AGB1, using transcriptome analyses on specific signals like ABA (Pandey et al. 2010), jasmonic acid (Okamoto et al. 2009), ozone (Booker et al. 2012) and *Plectosphaerella cucumerin* infection (Delgado-Cerezo et al.

2012). In addition, genome-wide interactome analysis of various G-protein subunits has been reported in *Arabidopsis* (Klopffleisch et al. 2011). Functional genomics and *in silico* studies have also begun to answer the doubts regarding the role of GCR1 in plant heterotrimeric G-protein signalling (Chakraborty et al. 2015; Taddese et al. 2014). However, we neither know the complete GPA1-mediated signaling pathway for the regulation of a single gene/process, nor the total number of genes/processes regulated by GPA1 on an organism-wide scale, let alone those shared by GCR1. This paper is an attempt to study the genome-wide role of GPA1 by isolating its knock-out mutant and analysing its transcriptome, as well as comparing it with our similar analysis of the role of GCR1 (Chakraborty et al. 2015), and with other transcriptome analyses on GPA1 (Booker et al. 2012; Delgado-Cerezo et al. 2012; Okamoto et al. 2009; Pandey et al. 2010).

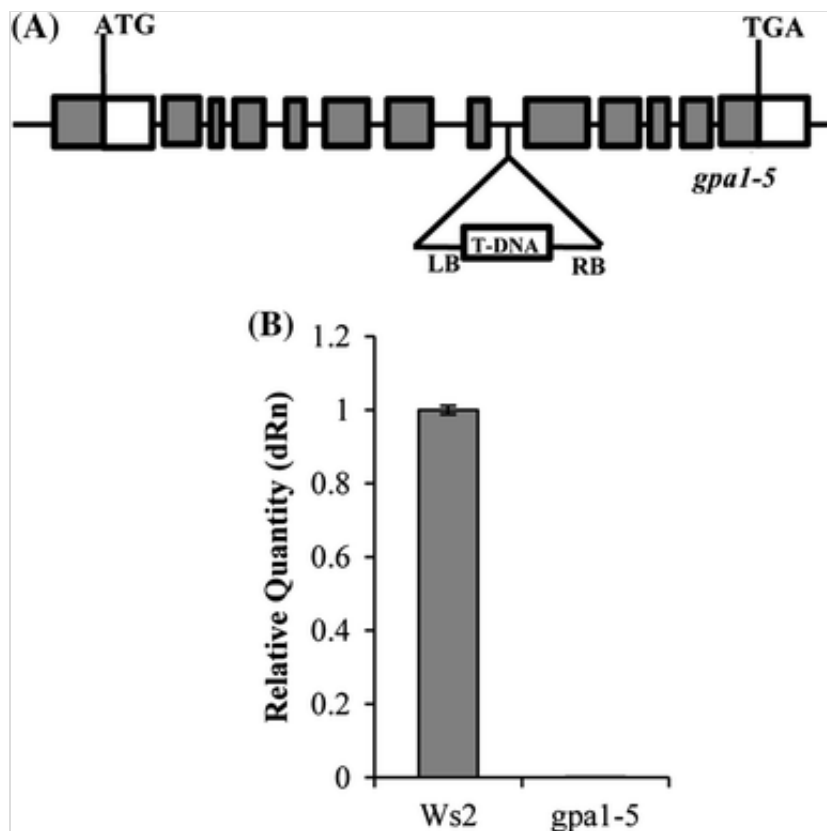
Results

GPA1 mutant characterization

The mutant was confirmed to have a single T-DNA insertion in the eighth intron of GPA1 (Fig. 1 a) and was designated as *gpa1-5*. Reverse transcriptase qPCR with gene-specific primers showed no expression of the GPA1 transcript, confirming that this is a knock-out mutation (Fig. 1 b). The mutant plants were phenotypically characterized for root length, plant height, leaf shape, etc. It was found that *gpa1-5* is similar to other known GPA1 mutants (Chen et al. 2004; Jones et al. 2003) with longer roots, fewer lateral roots, rounded leaves, reduced plant height, longer but fewer siliques, and smaller rosette (Supplementary Fig. S1) as compared to the wild type, Ws2 (Chakraborty et al. 2015).

Fig. 1

a(A) T-DNA insertion site/orientation in the *gpa1-5*. The exons are represented as *boxes* and the introns are represented as *lines*. LB and RB represent the *left* and *right border* respectively. **b(B)** Validation of the *gpa1-5* knockout mutation using qPCR, showing. ~~The mutant has shows~~ no expression of GPA1. The data presented is the average of three independent replicates \pm SE



Microarray analysis and validation

The microarray experiment was MIAME compliant and the high correlation coefficients across datasets (>0.9) clearly indicate the robustness and a high level of reproducibility of the data (Supplementary table S1). A stringent cut-off value of 1.0 (geometric mean \log_2) with a p value of ≤ 0.05 was used for determining the up- or down-regulated genes in the mutant with respect to the wild type control. The Benjamini Hochberg FDR procedure at a cut-off value of $p \leq 0.05$ was used for multiple testing correction. A total of 497 differentially regulated transcripts were obtained in the mutant (249 up-regulated and 248 down-regulated). These transcripts corresponded to 394 unique differentially expressed genes (DEGs) in the mutant (202 up-regulated and 192 down-regulated). A list of top 20 up- and down-regulated genes is shown in Table 1. A heatmap of all the differentially regulated genes is shown in Fig. 2a. In order to validate the microarray results, genes (9 up- and 7 down-regulated) from each category were selected and were subjected to RT-qPCR using gene specific primers checked for efficiency (90–100 %). The list of these genes and their primer sequences are given in the Supplementary table S2. The results of RT-qPCR matched with the microarray data in all the cases (Fig. 3) with Pearson's product moment correlation of >0.98 (p value = $2.66e-12$), validating the basic

trends of regulation of gene expression found on the microarray.

Table 1

List of the top 20 ~~each~~-up-regulated genes and the 20 most down-regulated genes in *gpa1-5* mutant

Locus id	Accession id	Gene name	Gene description	Log2FC	<i>P</i> val
Up-regulated in <i>gpa1-5</i>					
AT4G13920	NM_117466	AtRLP50	Receptor like protein 50	6.57	8.5 -0.
AT3G14710	NM_112332	AT3G14710	RNI-like superfamily protein	5.15	3.3 -0.
AT5G48430	NM_124218	AT5G48430	Eukaryotic aspartyl protease family protein	4.43	1.6 -0.
AT2G41240	NM_129689	BHLH100	Basic helix-loop-helix protein 100	4.24	6.0 -0.
AT1G14315	NM_001160864	AT1G14315	F-box and associated interaction domains-containing protein	4.03	3.9 -0.
AT2G06002	NR_022465	AT2G06002	Other RNA	3.95	2.3 -0.
AT5G64100	NM_125806	AT5G64100	Peroxidase superfamily protein	3.85	3.2 -0.
AT3G56970	NM_115556	BHLH038	Basic helix-loop-helix protein 38	3.45	5.9 -0.
AT3G05727	NM_001035568	AT3G05727	Encodes a defensin-like (DEFL) family protein	3.07	3.5 -0.
AT2G14610	NM_127025	PR1	Pathogenesis-related gene 1	3.04	2.9 -0.
AT1G68270	NM_105498	AT1G68270	AMP-dependent synthetase and ligase family protein	3.00	7.1 -0.

AT5G11140	NM_121152	AT5G11140	Arabidopsis phospholipase-like protein (PEARL14) family	2.99	7.7 -0.
AT1G70440	NM_105712	SRO3	Similar to rcd one 3	2.90	6.7 -0.
AT2G36690	NM_129224	AT2G36690	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2.60	1.2 -0.
AT5G60610	NM_125454	AT5G60610	F-box/RNI-like superfamily protein	2.55	1.2 -0.
AT1G47395	NM_179449	AT1G47395	Unknown protein	2.53	1.7 -0.
AT4G22520	NM_118378	AT4G22520	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.49	4.4 -0.
AT4G29370	NM_119082	AT4G29370	Galactose oxidase/kelch repeat superfamily protein	2.48	8.9 -0.
AT4G29760	NM_119122	AT4G29760	Unknown protein	2.47	2.4 -0.
Down-regulated in <i>gpa1-5</i>					
AT1G04890	NM_100367	AT1G04890	Unknown protein	-7.96	0.0
AT5G50300	NM_124409	AT5G50300	AZA-Guanine resistant2	-6.95	0.0
AT5G10880	NM_121126	AT5G10880	tRNA synthetase-related/tRNA ligase-related	-6.60	0.0
AT4G40100	NM_120176	AT4G40100	PP1 regulatory subunit2-like protein1	-6.18	0.0
AT2G38900	NM_129447	AT2G38900	PR-6 proteinase inhibitor family	-5.91	0.0

			protein		
AT2G30810	NM_128634	AT2G30810	Gibberellin-regulated family protein	−5.90	0.0
AT3G32150	NM_114038	AT3G32150	Unknown protein	−5.83	0.0
AT3G26250	NM_113532	AT3G26250	Cysteine/histidine-rich C1 domain family protein	−5.69	0.0
AT4G15750	NM_117666	AT4G15750	Plant invertase/pectin methylesterase inhibitor superfamily protein	−5.60	0.0
AT5G48350	NM_124210	AT5G48350	Polynucleotidyl transferase, ribonuclease H-like superfamily protein	−5.34	0.0
AT5G47350	NM_124106	AT5G47350	Alpha/beta-hydrolases superfamily protein	−5.30	0.0
AT3G58190	NM_115681	LBD29	Lateral organ boundaries-domain 29	−5.30	0.0
AT5G39260	NM_123288	ATEXPA21	Expansin 21	−5.15	0.0
AT4G35680	NM_119733	AT4G35680	Arabidopsis protein of unknown function (DUF241)	−4.97	0.0
AT5G24250	NM_122331	AT5G24250	Unknown protein	−4.95	0.0
AT1G30020	NM_102742	AT1G30020	Protein of unknown function, DUF538	−4.92	0.0
AT3G24510	NM_113361	AT3G24510	Defensin-like (DEFL) family protein	−4.91	0.0
AT4G21830	CD530941	MSRB7	Methionine sulfoxide reductase B7	−4.84	0.0
AT1G32020	NM_102936	AT1G32020	F-box family	−4.83	0.0

			protein		
AT1G54445	NM_001036113	AT1G54445	Defensin-like (DEFL) family protein	−4.80	0.0

Fig. 2

a Heat map of differentially expressed genes. The background-subtracted microarray data were subjected to hierarchical clustering using Genespring software ver. 11.5 to generate the heatmap. *Yellow* represents the control data, while *red* and *green* represent up-regulation and down regulation respectively. **b** GO categorization of DEGs. The DEGs were categorized into GO classes using classification supervisor tool of Bioarray resource (www.bar.utoronto.ca)

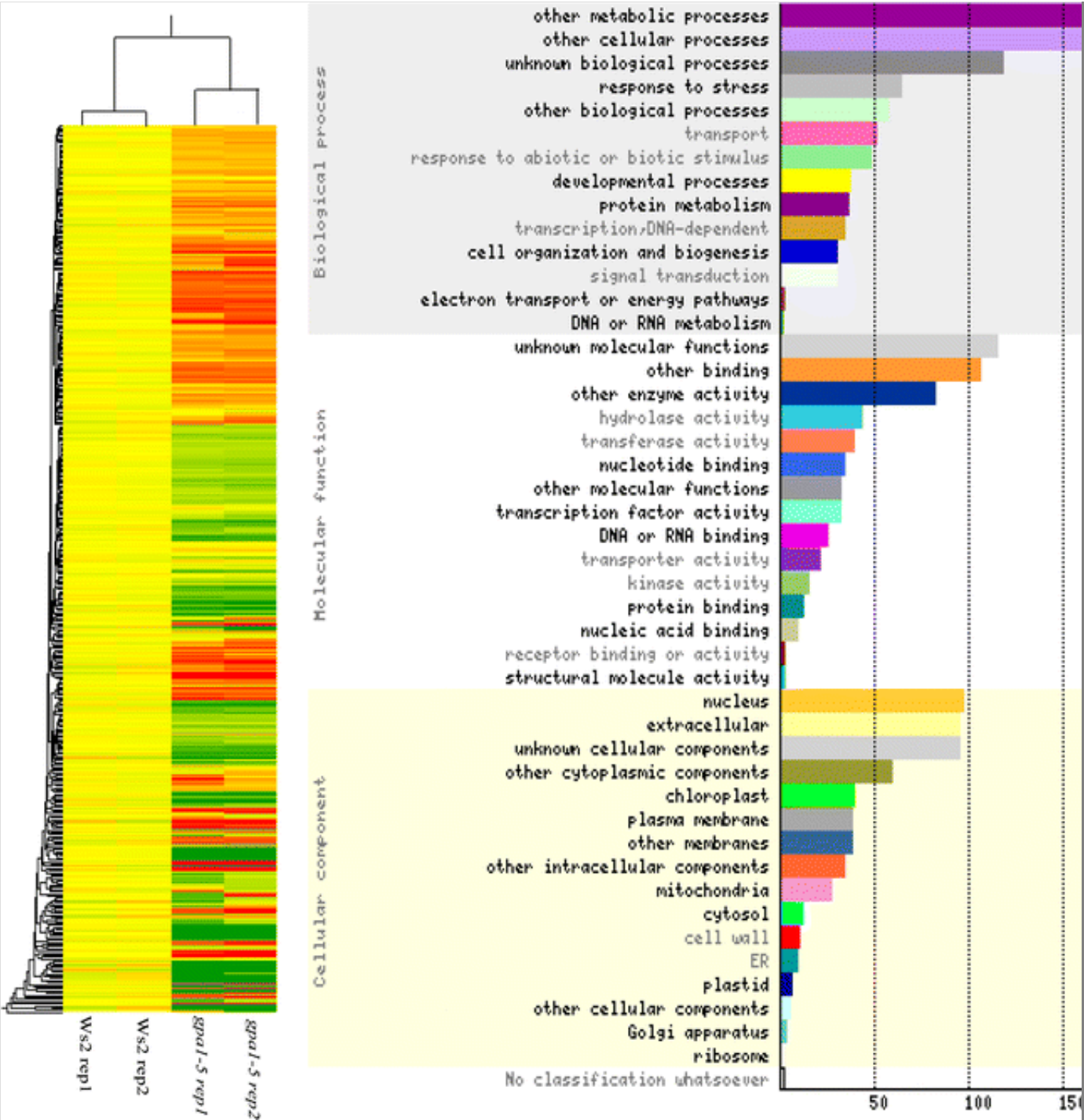
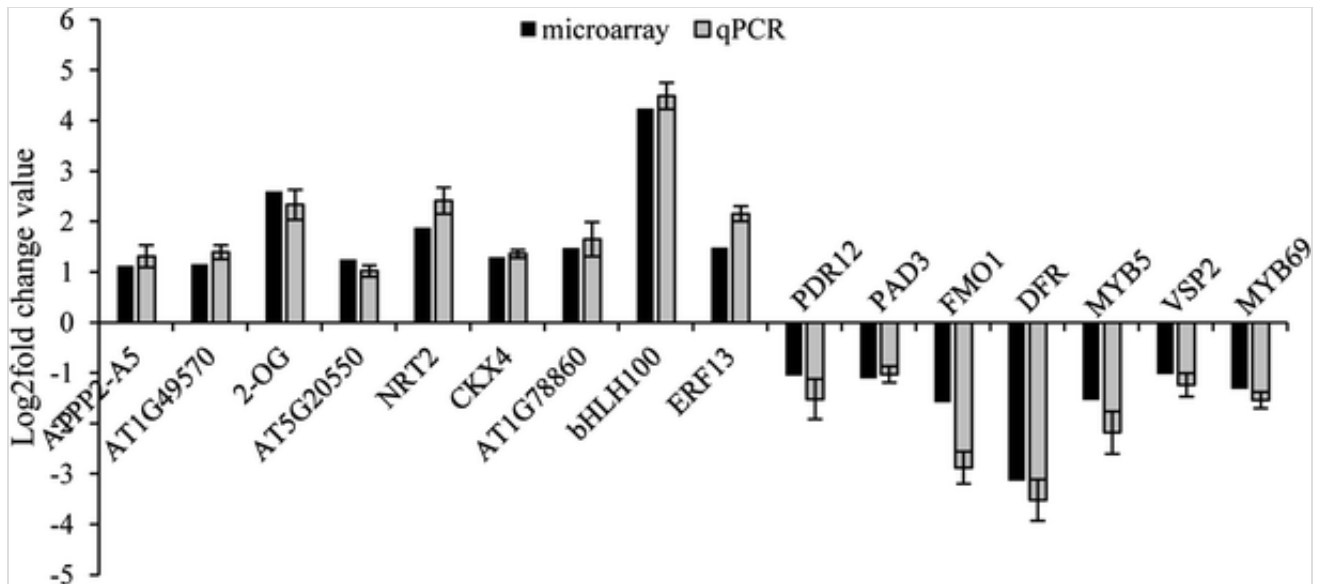


Fig. 3
qPCR validation of selected DEGs and comparison with microarray of few DEGs data in *gpa1-5* mutant. The real time RT-PCR was performed using biological triplicates. The values are represented as log2fold change \pm SE. AT1G49570: Peroxidase superfamily protein; AT5G20550: 2-oxoglutarate; AT1G78860: curculin-like (mannose-binding) lectin family protein



AQ1

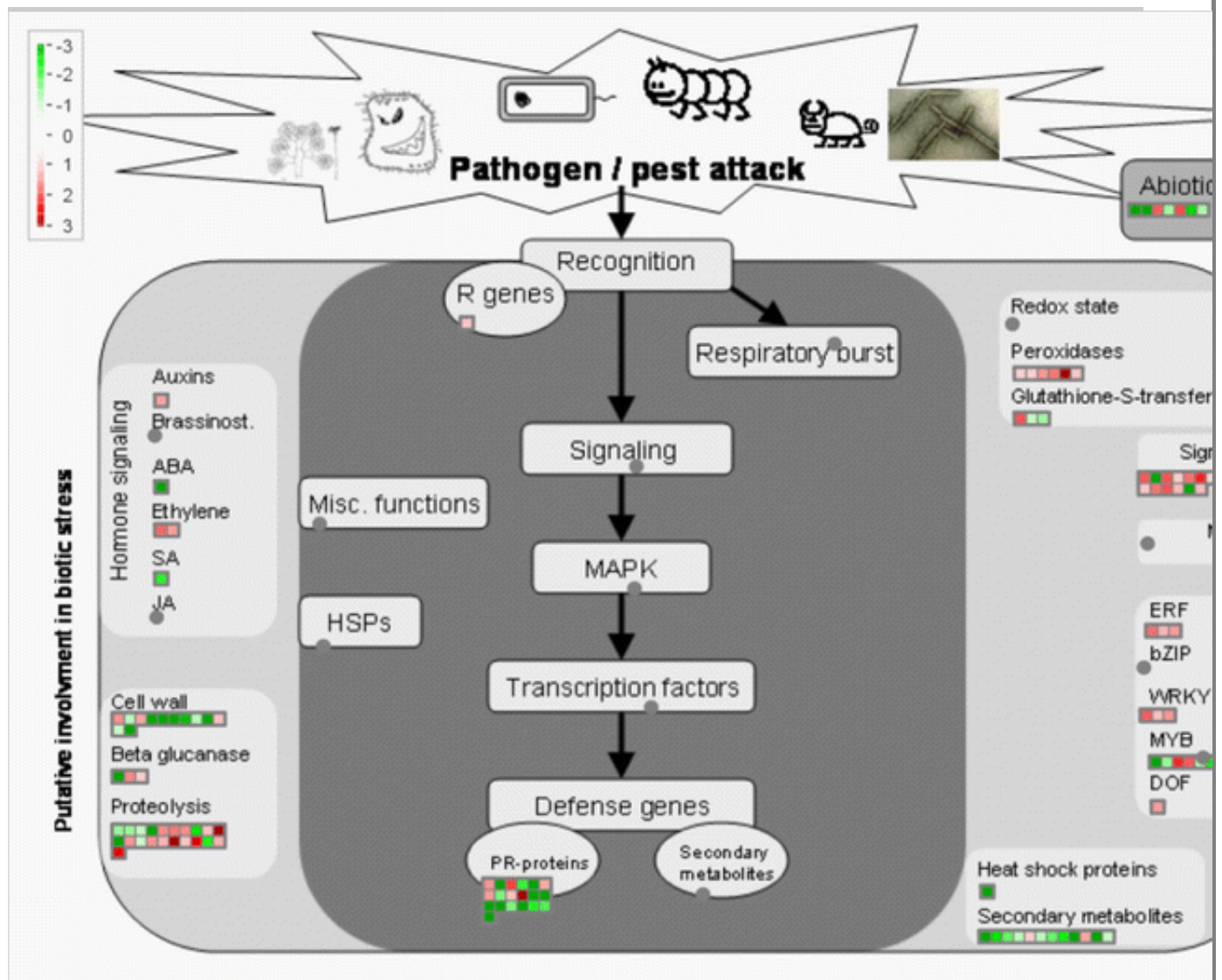
Biotic and abiotic stress

Several functional analyses of the differentially expressed genes (DEGs) using gene ontology (GO), singular enrichment analysis (SEA) and Mapman (Fig. 2b, Supplementary table S3, Fig. 4) revealed stress response as one of the major categories with 65 genes (41 up/24 down) based on SEA, constituting 16.5 % of the total GPA1 response. While nearly half of them are known to be GPA1-regulated (Supplementary table S5), such as pathogenesis-related protein 1 (*PR1*), Vegetative storage protein 2 (*VSP2*), Dihydroflavonol 4-reductase (*DFR*), etc. (Booker et al. 2012; Okamoto et al. 2009; Pandey et al. 2010), our analysis reveals the role of GPA1 in regulating the remaining stress-responsive genes. They include some well-known stress response genes like Yellow leaf-specific gene 9 (*YLS9*), Plant U-box 22 (*PUB22*), several peroxidases and transcription factors, etc. The basic trends of their regulation in the mutant have been confirmed by qRT-PCR on two up-regulated genes (peroxidase family protein gene (*AT1G49570*) and *ATPP2A5*) and two down-regulated ones (*PDR12* and *PAD3*), as shown in Fig. 3. Our Mapman analysis identified 119 genes out of 394 in the biotic stress category, which includes a few genes that are also responsive to abiotic stress. A majority of these 119 genes were mapped into PR-proteins, signalling, secondary metabolism, proteolysis and cell wall (Fig. 4). We also found several genes coding for expansins, pectinesterase, methylpectinesterase etc., which are involved in cell wall modification, indicating the convergence of some aspects of biotic stress with development in

GPA1-response.

Fig. 4

Distribution of DEGs ~~into~~^{under} the biotic stress category according to Mapman. 119 genes out of 394 were mapped into this category. The differential regulation is according to the scale given, *red* represents up-regulation while *green* represents down-regulation in *gpa1-5*



Development/morphogenesis

At least 37 DEGs (17 up/20 down) are involved in morphogenesis and development (Supplementary table S3), such as the genes for male sterility (*MS2*), transcription factor *MYB5*, genes for maternal effect embryo arrest (*MEE48*), LOB domain containing protein (*LBD*), various expansin subunits

etc. Out of these 37, 8 genes have also figured in the raw data of earlier studies on GPA1 mutant transcriptomes, though they were not specifically reported to be GPA1-regulated (Supplementary table S5). Nevertheless, they indicate their consistency with our results. The validation of our data by qRT-PCR confirmed the upregulation of *CKX4* and a lectin family protein (AT1G78860) and downregulation of *MYB5* and *VSP2* (Fig. 3). A key gene downregulated in the *gpa1-5* mutant is *LBD29*, which is associated with lateral root development. An additional set of 18 DEGs identified in the mutant belong only to development and not morphogenesis, such as the genes for LEA domain-containing protein, Transparent Testa 12 (*TT12*), *DELTA-VPE*, etc., that are involved in the development of fruit and seed. SEA revealed several other genes involved in aging, such as vegetative storage protein 2 (*VSP2*), Dark inducible protein 11 (*DIN11*), Senescence associated gene (*SAG13*), Escarola (*ESC*), Yellow leaf-specific gene 9 (*YLS9*), etc.

Secondary metabolism/flavonoid biosynthesis

Pathway analysis of all DEGs revealed that flavonoid biosynthesis is one of the most significant GPA1-responsive pathways, as shown in Table 2. Mapman analysis also revealed 13 DEGs (2 up/11 down) involved in secondary metabolite biosynthesis, mainly flavonoid biosynthesis (Fig. 5). They include genes encoding flavanone 3- β -hydroxylase, dihydroflavonol-4-reductase, flavonoid 3'-monooxygenase, flavonol synthase, etc. Most of these were found to be down-regulated, while only a few were up-regulated. The trend of regulation were confirmed by qRT-PCR of two up-regulated (*2-OG* and AT5G20550) and two down-regulated genes (*FMO1* and *DFR*). Out of the 13 DEGs, 7 genes have also figured in the earlier studies on *gpa1* mutant transcriptomes, though they were not specifically reported to be GPA1-regulated (Supplementary table S5). Nevertheless, they indicate their consistency with our results on the potentially important role of GPA1 in regulating secondary metabolism.

Table 2

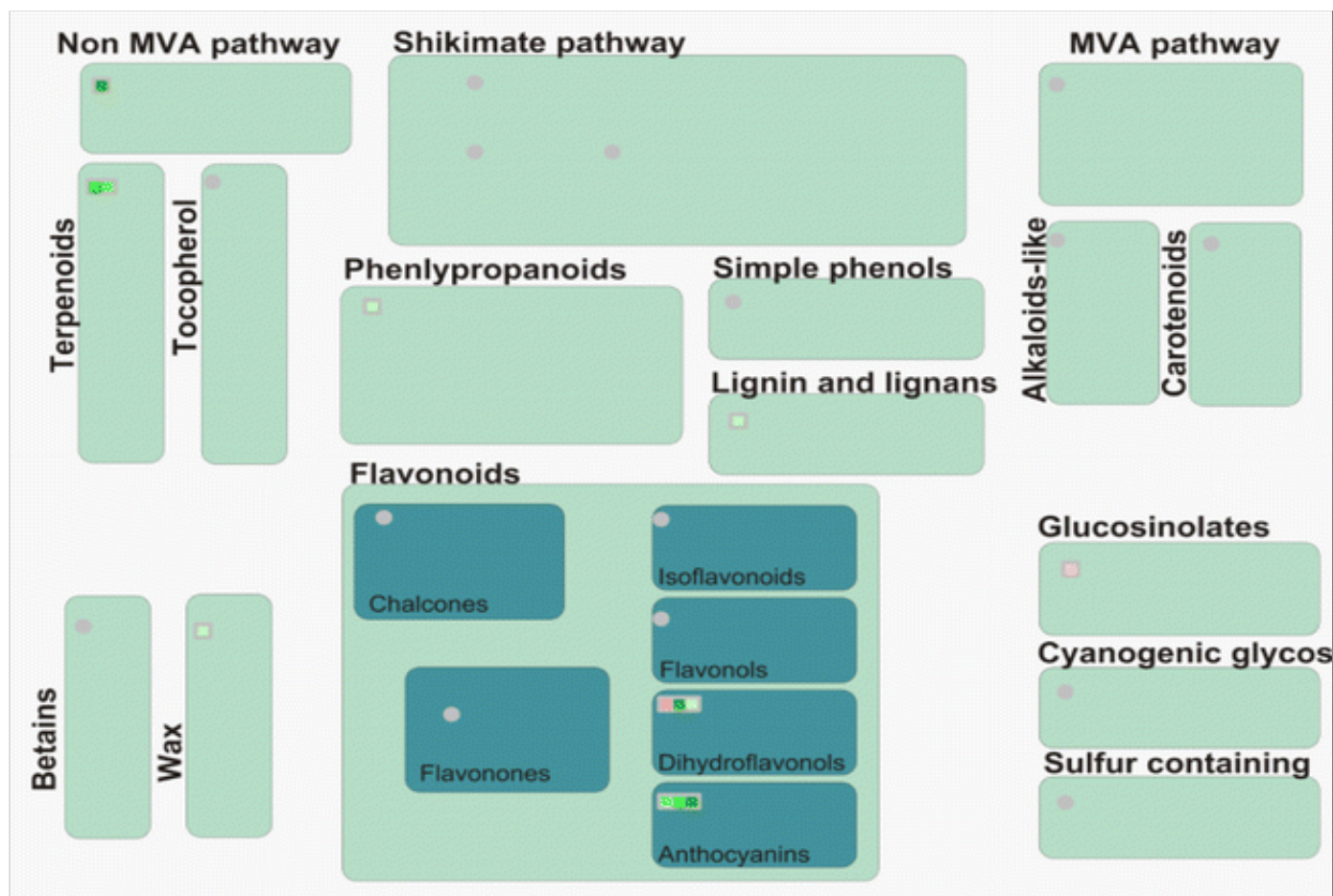
List of changed pathways in the mutant

Rank	Changed pathway	<i>p</i> value
1	Leucodelphinidin biosynthesis	0.003796

2	Leucopelargonidin and leucocyanidin biosynthesis	0.003796
3	Flavonol biosynthesis	0.005997
4	Coniferin metabolism	0.006383
5	Monolignol glucosides biosynthesis	0.006383
6	2,3-cis-flavanols biosynthesis	0.021443
7	Pyruvate fermentation to ethanol II	0.02588
8	Acetaldehyde biosynthesis I	0.02588
9	Gibberellin inactivation II (methylation)	0.042435
10	Superpathway of flavones and derivatives biosynthesis	0.043732
11	Luteolin biosynthesis	0.062988
12	Asparagine biosynthesis I	0.062988
13	Camalexin biosynthesis	0.062988
14–44	Others	>0.063
Pathways significantly (p value ≤ 0.05) altered in the mutant are represented in bold		

Fig. 5

Distribution of DEGs ~~into~~^{under} the secondary metabolism category according to Mapman. The differential regulation is according to the scale given, *red* represents up-regulation while *green* represents down-regulation in *gpa1-5*



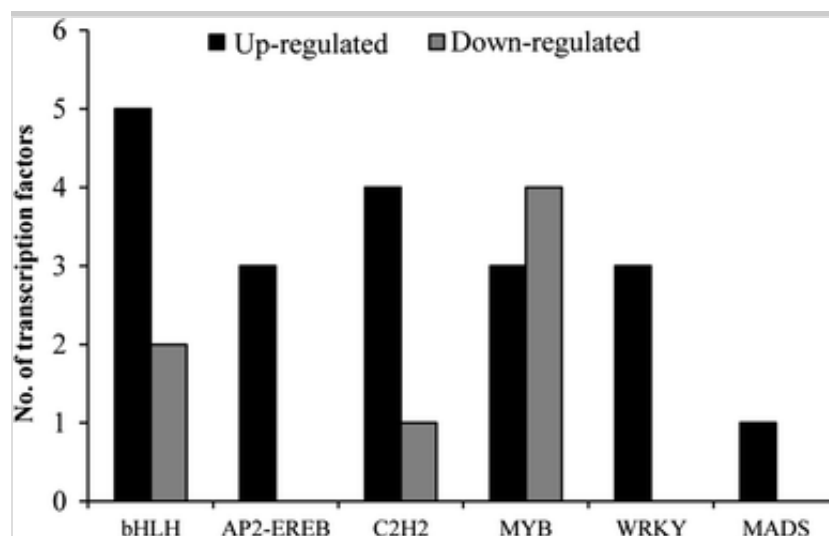
Transcription factors

As many as 40 GPA1-responsive DEGs (28 up/14 down) have been found to belong to transcription factors from 18 families (other than the putative and unspecified ones) listed in the plant transcription factor database (PlantTFDB 2.0). Their validation by qRT-PCR confirmed the upregulation of *bHLH100* and *ERF13*, and downregulation of *MYB5* and *MYB69* (Fig. 3). The full list of all the transcription factor genes and families and the mode of their regulation (up/down) is provided in Supplementary Table S4. The highest represented families among them are bHLH, AP2-EREB, C2H2, MYB and WRKY (Fig. 6). Most of the members of these families were up-regulated, with fewer downregulated genes. However, in the case of AP2-EREB, WRKY and MADS families, there was only upregulation and no case of down-regulation. Interestingly, a transcription factor involved in phosphate response, *WRKY75*, was found to be down-regulated in the *gpa1-5* mutant. It was not shown in Fig. 6, as its Log₂ fold-change value was slightly beyond the cut-off at -0.9, even though its *p* value was highly significant at 0.003. Overall out of these 40 DEGs coding for transcription factors, 7 genes have also figured in the earlier

studies on GPA1 mutant transcriptomes, though they were not specifically reported to be GPA1-regulated (Supplementary table S5). Nevertheless, they indicate their consistency with our results on the potentially important role of GPA1 in the transcriptional regulation of their target genes.

Fig. 6

Distribution pattern of transcriptions factors into the highly represented TF families. This classification was based on plant transcription factor database (plantTFDB) (Zhang et al. 2010)



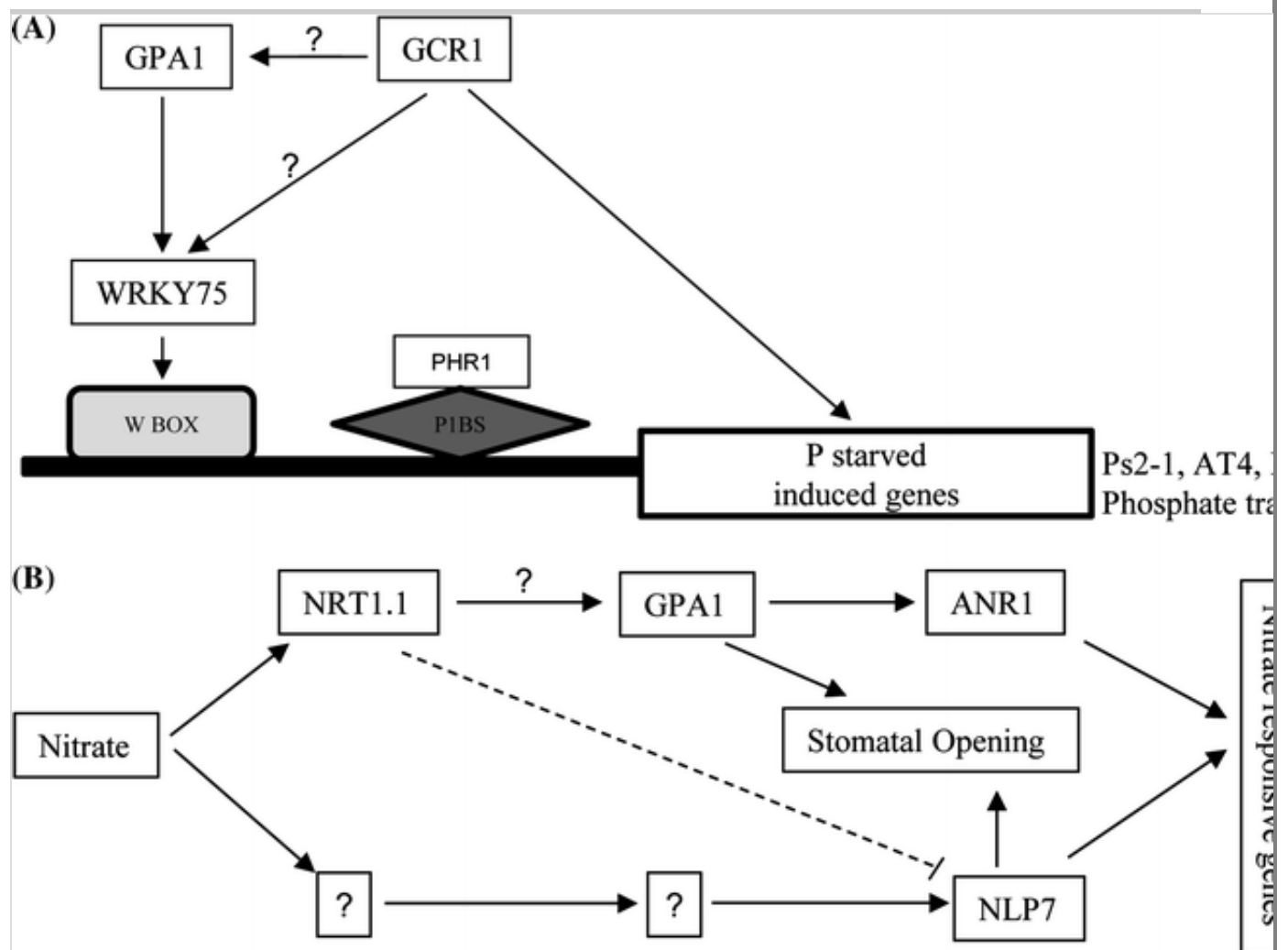
Transporters and nutrient response

Transport also emerged as one of the major categories of GPA1-responsive genes, with 51 DEGs (34 up/17 down) (Supplementary table S3). They include several lipid transfer proteins (LTPs), nitrate transporter (*NRT2.1*), phosphate transporter (*PHT1.1*), methylammonium transporter (*ATTIP2.3*), MATE efflux family protein and *PDR12*, both involved in multidrug transport. The differential regulation of *NRT2.1* and *PDR12* were verified using qRT-PCR (Fig. 3). Several genes involved in nitrogen starvation/assimilation were also found to be up-regulated in *gpa1-5*, such as nitrate transporter (*NRT2.1*), isocitrate dehydrogenase (*ICDH*), asparagine synthase (*ASN1*), etc. In addition, certain nutrient and other stress-related gene categories such as peroxidases, kinases and cytochrome P450-s were also found to be differentially regulated in the *gpa1-5* mutant. A schematic of probable involvement of G-protein in phosphate and nitrate response is shown in Fig. 7. Interestingly, none of the earlier studies on GPA1 mutant transcriptomes specifically reported any nutrient

transporter or nutrient response to be GPA1-regulated, even though *NRT2.1* and *PDR12* were found in their raw data (Supplementary table S5).

Fig. 7

Schematic showing probable involvement of GPA1 in **a** phosphate response and **b** nitrate response. *Arrows* show the actual direction of regulation. *Dotted line* represents negative regulation? Represents the intermediates or steps which are yet to be tested or identified



Correspondence with other GPA1 mutant transcriptome data

In addition to finding several genes corresponding to the above functional categories unreported in others' studies, we compared our overall transcriptome data with all the 4 lists of DEGs available at GEO and Arrayexpress from published transcriptome studies on GPA1 mutants by others (Booker et al.

2012; Delgado-Cerezo et al. 2012; Okamoto et al. 2009; Pandey et al. 2010) (Fig. 8). The total number of DEGs reported in them ranged from 307 (Okamoto et al. 2009) to 656 (Pandey et al. 2010), with ours in between at 394. Venn selection between all of them revealed that our dataset was a part of all combinations that yielded the highest number and/or percentage of overlapping genes (17 with any 4 studies, 33 with any 3 studies and 52 with any 2 studies), whereas the dataset of E-MEXP-1822 (Okamoto et al. 2009) yielded the least number of overlaps in every combination. The similarities in some of the broad functional categories of genes are more striking, as shown in Table 3. Stress response emerged as the largest annotated category of genes differentially regulated in the GPA1 mutant in all the 5 studies including ours, followed by transcription factors, whereas development/morphogenesis, secondary metabolism and transport/nutrient responses varied between the next three positions in different studies. These results indicate the gross similarity in the overall process categories of the GPA1-regulated genes we identified from different studies. A comparison of individual DEGs and their up/down regulation between different studies including ours is shown in Supplementary Table S5. The data indicate the high level of correspondence in the regulation of individual genes between our data and those of others. We found 90 common DEGs to be identically regulated (up/down) between all the above studies and ours. Out of these, 52 genes are common with those obtained from GSE 34667 (Booker et al. 2012), 49 genes are common with GSE 19520 (Pandey et al. 2010), 47 common with E-MEXP-3733 (Delgado-Cerezo et al. 2012) and 10 genes common with E-MEXP-1822 (Okamoto et al. 2009). The genes common to all include alternative oxidase, *PDR12*, *PAD3*, *ATPP2-A5*, *WRKY46*, *PR1*, *YLS9*, *SAG13*, *DIN11*, etc. with similar trend of differential regulation (Supplementary Table S5). Overall, these results clearly indicate that GPA1 has far more extensive genomewide roles than has been reported so far from signal-specific or response-specific studies.

Fig. 8

Venn selection of DEGs in different transcriptomes. This Venn diagram shows the overlap of our GPA1 mutant transcriptome data with those others. The data of Okamoto et al. (2009) has been excluded due to minimal overlap with any of the data

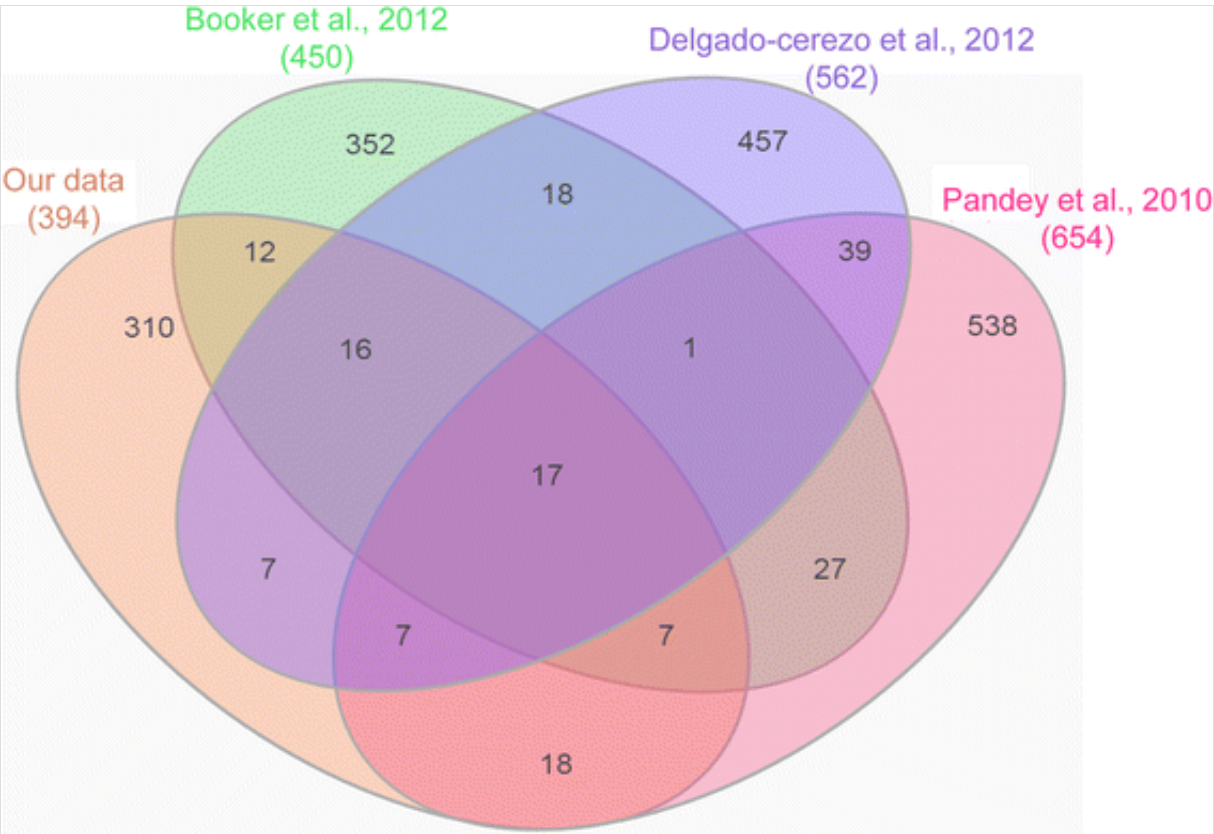


Table 3
Distribution of GPA1-regulated genes from different studies into functional categories

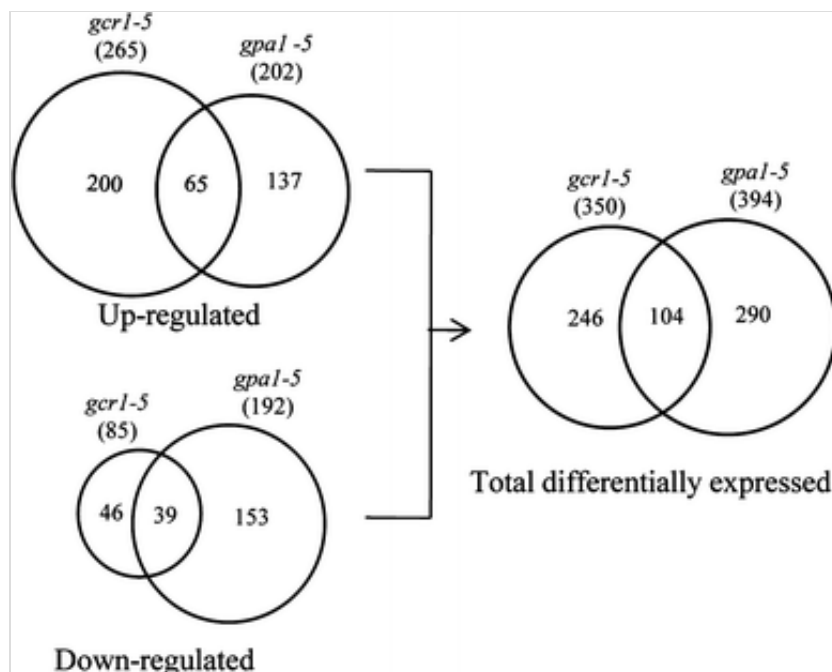
Biological process	Our data		Pandey et al. (2006, 2009, 2010)		Booker et al. (2012)	
	No. of genes	<i>p</i> value	No. of genes	<i>p</i> value	No. of genes	<i>p</i> value
Abiotic/biotic stress	65	1.5e−21	182	4.36e−31	125	4.37e−31
Development/morphogenesis	28	8.1e−10	67	0.000786	20	0.038
Secondary metabolism/flavonoid biosynthesis	15	0.00011	29	1.93e−06	26	4.02e−06
Transcription factors	30	1.7e−19	69	4.07e−04	32	0.000
Transport and nutrient reponse	23	2.8e−07	28	60.3e−06	15	0.001

Comparison of GPA1 and GCR1 responses

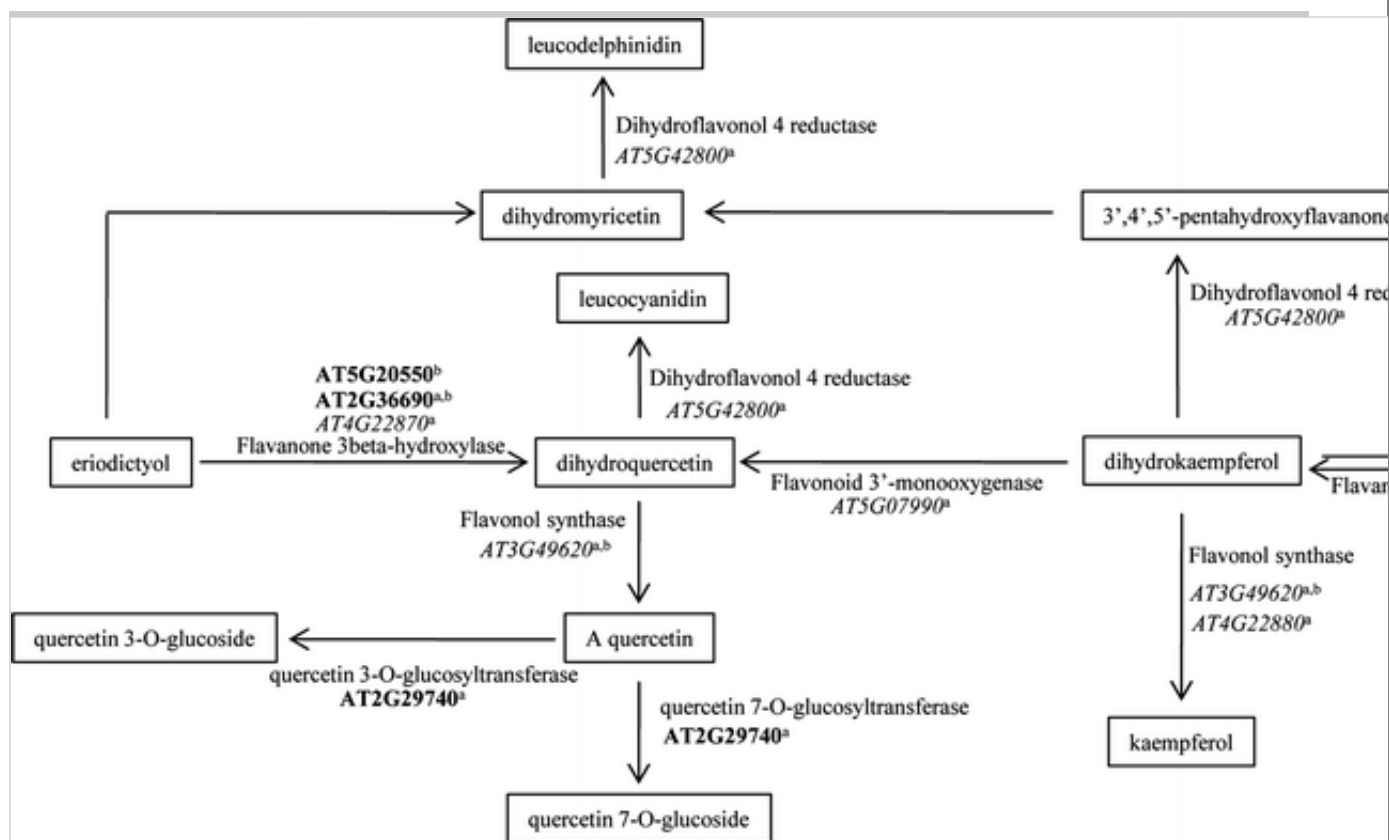
We compared the GPA1 responsive genes identified in the present study with GCR1-responsive genes we identified in a simultaneous transcriptome analysis of a GCR1 mutant under identical experimental conditions (Chakraborty et al. 2015). We found that 104 genes (65 up- and 39 down-regulated) were common to GPA1 and GCR1 (Fig. 9 and Supplementary table S6). They constitute 26 and 30 % of the total GPA1-responsive and GCR1-responsive genes identified in our microarray studies. These data clearly show that even though a majority of the DEGs in both the mutants are uniquely regulated by either GCR1 or GPA1, a significant minority are regulated identically by both. Our SEA analysis of the genes common in both GCR1 and GPA1 transcriptomes (Supplementary table S7) revealed that they belong to categories like kinases, phosphatases, abiotic stress response, chitin response, flavonoid biosynthesis, transcription factors, etc. When we compared the DEGs from both the mutants at the pathway level using plantMetGenMap (using AraCyc as background) we found that both GCR1 and GPA1 are involved in the regulation of flavonoid and its derivatives (Fig. 10). Interestingly, even though GCR1 regulates fewer genes than GPA1 in this pathway, those of them regulated by both have a similar trend of up/down regulation (Fig. 10). This is further validated by the qRT-PCR data of the 16 GPA1-regulated genes shown in Fig. 3. Their comparison with the RT-PCR data on the 17 GCR1-regulated genes reported elsewhere (Chakraborty et al. 2015) confirms that 8 genes are regulated identically (up/down) in both the mutants, while 8 and 9 genes are uniquely regulated in the *gpa1-5* mutant and *gcr1-5* mutant respectively.

Fig. 9

Venn selection of DEGs in *gcr1-5* and *gpa1-5*. The Venn selection was carried out using online Venn selection tool of BioinfoRx. The detailed analyses of ~~ger1~~*GCR1* mutant (*gcr1-5*) transcriptome data have been published separately (Chakraborty et al. 2015)

**Fig. 10**

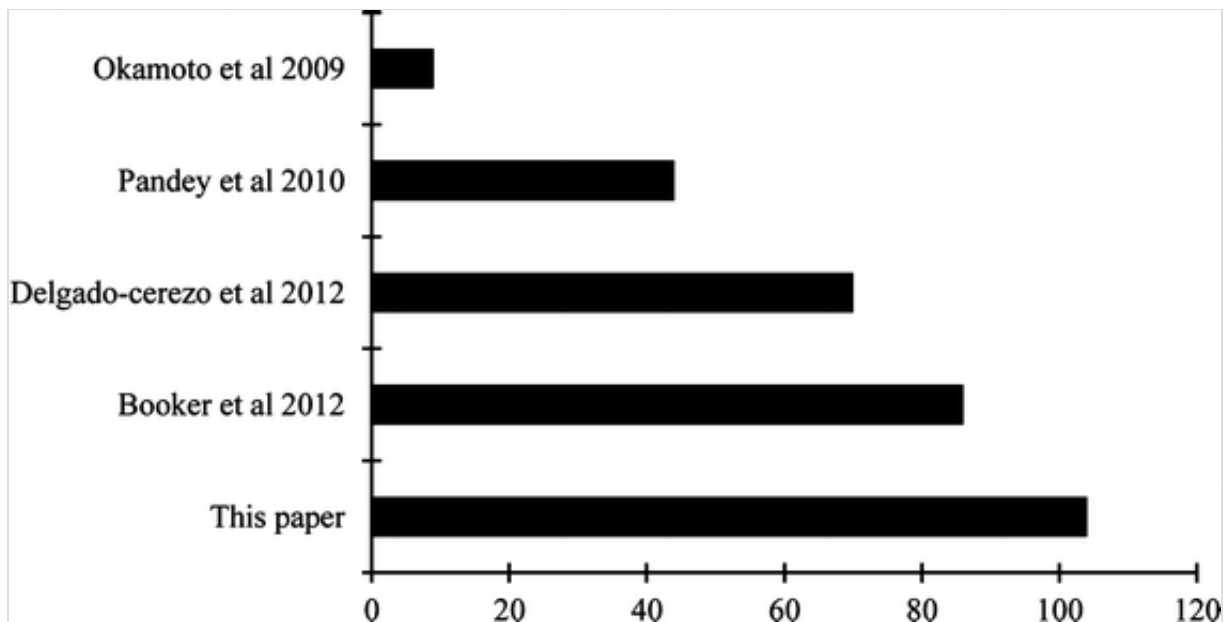
Superpathway of flavonoids and derivatives. Genes marked in *bold* are up-regulated while those in *italics* are down-regulated. ^aDEG in *gpal-5*. ^bDEG in the GCR1 mutant



We also compared the DEGs from other GPA1 mutant transcriptomes (Booker et al. 2012; Delgado-Cerezo et al. 2012; Okamoto et al. 2009; Pandey et al. 2010) with our GCR1-responsive DEGs (Chakraborty et al. 2015). Out of a total of 114 common genes, 86 were common to GSE 34667 (Booker et al. 2012), 70 common to GSE 19520 (Coursol et al. 2003), 44 common to E-MEXP-3733 (Pandey et al. 2006) and 9 common to E-MEXP-1822 (Okamoto et al. 2009) in addition to the 104 common genes to our data (Fig. 11). The common genes include *PDR12*, *PRI*, *DIN11*, *WRKY29*, *GLIP1*, etc. The GO processes regulated by these include response to stress, transport, signal transduction, etc. The similarities in some of the broad functional categories of genes are more striking, as shown in Table 4. Stress response emerged as the largest annotated category of GCR1-responsive genes differentially regulated in the GPA1 mutant in all the 5 studies including ours, followed by transcription factors and secondary metabolism/flavonoid biosynthesis in different studies. Interestingly, the correspondence between the GCR-1-regulated and GPA1-regulated functional categories is far higher when the total list of DEGs in each transcriptome study is compared, rather than comparing the DEGs common to different transcriptome studies (data not shown). This could be due to the usual differences between various studies at the level of individual genes, even if they belong to the same pathways. Overall, these results indicate the gross similarity in many of the broad process categories regulated by GCR1 and GPA1, not only in our own studies but also from others, strongly indicating the possibility of GCR1–GPA1 coupling in the regulation of these genes/processes.

Fig. 11

Comparison of shared DEGs with GCR1. The *bars* show the total number of DEGs in each of the GPA1 transcriptome paper shared with those of GCR1

**Table 4**

Distribution of GPA1-regulated genes overlapping with DEGs of GCR1 mutant into functional categories

Biological process	DEGs identified in the GCR1 mutant transcriptome study of Chakraborty et al. (2015)		Genes common to the DEGs identified GPA1 mutant				
			Our data		Booker et al. (2012)		Del Celis (2006)
	No. of genes	<i>p</i> value	No. of genes	<i>p</i> value	No. of genes	<i>p</i> value	No. of genes
Abiotic/biotic stress	75	7.8e-40	56	1.56e-21	36	8.2e-14	36
Hormone response/biosynthesis	5	0.00051	n.a	n.a	n.a	n.a	8
Secondary metabolism/flavonoid biosynthesis	6	0.00034	15	0.00011	n.a	n.a	n.a
Transcription factors	31	9e-22	30	1.7e-19	5	0.00075	5

Discussion

G-protein signalling pathways have been found to be involved in a large number of plant processes, mainly obtained through pharmacological studies and molecular genetic analyses of various loss-of-function mutants and gain-of-function overexpression lines (Urano et al. 2013). Nevertheless, we are far from understanding their organism-wide role in plants, partly because of the limitations of signal-specific or response-specific approaches and partly because the recent advances in plant genomics have not been fully exploited. In this paper, we explore the genes/processes/pathways regulated by GPA1 in *Arabidopsis* on a genome-wide scale using whole transcriptome microarray analysis of a knock-out mutant, *gpa1-5*, and also compare it with similar analyses by others on GPA1 as well as GCR1.

We isolated a novel knock-out mutant of GPA1 disrupted at the 8th intron (Fig. 1) and designated it as *gpa1-5*. Phenotypic characterization (Supplementary Fig. S1) confirmed that it is similar to other GPA1 mutants (Chen et al. 2004; Jones et al. 2003). Comparative whole transcriptome microarray analysis of the *gpa1-5* mutant and the corresponding wild type, Ws2, revealed 394 differentially expressed genes (DEGs), using a stringent cut-off of $\log_2\text{FC}$ 1.0 with a p value ≤ 0.05 . They span all five chromosomes (data not shown) with up- and down-regulated genes in equal proportion (51:49). Sixteen of them have been confirmed by qRT-PCR (9 up and 7 down) and a larger list of 20 each is given in Table 1.

A comparison of our transcriptome data with those of other GPA1 mutants (Booker et al. 2012; Delgado-Cerezo et al. 2012; Okamoto et al. 2009; Pandey et al. 2010) revealed three significant findings: Firstly, there are a number of genes common to these studies and our dataset figures in all combinations that yielded the best overlaps (Fig. 8). Secondly, the commonality in terms of functional categories is far higher (Table 3), considering that there were large number of genes not shared between any dataset. However, the dataset of E-MEXP-1822 (Okamoto et al. 2009) had very little in common with ours or any others (except to some extent with E-MEXP-3733 (Pandey et al. 2006)), presumably because of the smallest number of DEGs they found, or due to their experimental conditions in terms of age and part of plant used, light intensity, photoperiod, etc. (Supplementary table S8). Thirdly, neither the GPA1-regulation of these genes/processes, nor their commonalities with others were

specifically recognized or stated in the earlier studies (Booker et al. 2012; Delgado-Cerezo et al. 2012; Okamoto et al. 2009; Pandey et al. 2010), lending novelty to our findings. Overall, these results clearly indicate that GPA1 has far more extensive genomewide roles than has been reported so far from signal-specific or response-specific studies. Interestingly, while none of the genes coding for the 41 proteins reported to interact with GPA1 (Klopfleisch et al. 2011) were found to be differentially regulated in our study, two of their processes are shared (morphogenesis and cell wall modifications), perhaps because they are regulated at the protein level.

Abiotic and biotic stress

Stress response is one of the better studied aspects of GPA1 in plants (Pandey et al. 2006, 2010), but our transcriptome data adds two new dimensions to it. Firstly, we report 41 additional genes from the stress-response category (Supplementary Table S3), about half of them for the first time, while the rest figured only in the raw data of other GPA1 transcriptome studies (Booker et al. 2012; Delgado-Cerezo et al. 2012; Pandey et al. 2010), but remained unreported. Secondly, while abiotic stress dominates the literature on GPA1 (Colaneri et al. 2014; Pandey et al. 2006, 2010), our Mapman analysis clearly indicates biotic stress as an important GPA1-regulated functional category for the first time, with 119 genes out of a total of 394 DEGs (Fig. 4). A few of them, such as some peroxidases and cytochromes have been reported to be involved in G-protein-mediated oxidative stress response induced by ozone (Booker et al. 2012; Joo et al. 2005). A few others are known to be differentially regulated in *agb1* and *agg1/2* mutants, such as *Arabidopsis thaliana* phloem protein-A5 (*ATPP2-A5*), pathogenesis-related protein 1 (*PR1*), flavin-dependent monooxygenase 1 (*FMO1*), phytoalexin deficient 3 (*PAD3*) etc. (Liu et al. 2013; Nitta et al. 2014; Trusov et al. 2010). Thus, our study comprehensively captures the extensive role of GPA1 in stress response in general and biotic stress response in particular for the first time on a genomewide scale.

Development/morphogenesis

The role of G-proteins in development has been explored in a few physiological studies (Pandey et al. 2006; Ullah et al. 2003), but the genes involved in it

were not known. Our study revealed 37 genes related to development as one of the major GPA1-regulated categories, with roles mainly in the development of fruit and seed (Supplementary Table S3). They include 8 genes from the raw data of other GPA1 transcriptome studies (Booker et al. 2012; Delgado-Cerezo et al. 2012; Pandey et al. 2010) that went unreported. A few of the DEGs in this category are *MS2* (male sterility 2), *MEE48* (maternal effect embryo arrest 48), *DELTA-VPE* (vacuolar processing enzyme), *RTFL1* (rotundifolia like 1), *LBD29* (LOB domain containing protein 29) and various expansin subunits. Many of these development related proteins have been used in crop improvement (Dong et al. 2013). Of these, *LBD29* has been associated with lateral root development (Feng et al. 2012), and its downregulation in the *gpa1-5* mutant could be responsible for the reduced number of lateral roots observed in the mutant (data not shown). Some other DEGs like expansins are involved in cell wall modification during development (McQueen-Mason et al. 1992). Thus, our microarray data support the association of G-proteins with morphogenesis and cell wall modifications, based on a genomewide interactome study (Klopffleisch et al. 2011).

Secondary metabolism/flavonoid biosynthesis

In an indication of the importance of G-protein signalling in the regulation of secondary metabolic pathways, 11 genes of the flavonoid biosynthesis pathway are down-regulated in the *gpa1gpa1-5* mutant, while 2 are up-regulated (Fig. 5). The predominance of downregulation is evident from the finding that flavonoid levels were lowered in the guard cells of *Arabidopsis* GPA1 mutants, *gpa1-3* and *gpa1-4* (Jin et al. 2013). Our data from Fig. 5 clearly show that transcriptional downregulation could be the mechanism by which the flavonoid levels were lowered in the *gpa1* mutant (Jin et al. 2013). Out of the above 13 DEGs, 7 genes (Supplementary Table S5) have also figured in the raw data of other GPA1 transcriptome studies (Booker et al. 2012; Delgado-Cerezo et al. 2012; Pandey et al. 2010), though they were not specifically reported to be GPA1-regulated. They include enzymes of flavonoid biosynthesis like dihydroflavonol-4-reductase, flavonoid 3'-monooxygenase, which could be particularly important in plants like tea, in which flavonoids are of commercial relevance.

Transcription factors

Transcription factors are known to mediate stress responses (Dubos et al. 2010), guard cell function and root hair differentiation (Ramsay and Glover 2005), cell fate and metabolic regulation (Wu et al. 2005), as was the role of G-proteins in these processes (Colaneri et al. 2014; Ullah et al. 2001; Wang et al. 2001). However, the involvement of G-proteins in regulating these processes *via* these transcription factors was not known. Other GPA1 transcriptome studies did have some transcription factors in their GEO data, but did not claim their GPA1 regulation in their publications (Booker et al. 2012; Delgado-Cerezo et al. 2012; Pandey et al. 2010). Our study identified 40 transcription factors belonging to 18 families as GPA1-responsive, including AP2-EREB, C2H2, WRKY and MYB families (Supplementary Table S4). This is the most comprehensive, if not the first report on the GPA1-regulation of such a large number of transcription factors from diverse families with diverse functions. This also provides the means to approach stress signalling from either the G-protein end or the transcriptional end.

Transporters and nutrient response

Transmembrane transporters are another important category of GPA1-regulated genes found in our study (Supplementary Table S3). While a nitrate transporter (*NRT2.1*) and the pleiotropic drug resistance gene (*PDR12*) have been reported earlier in another *gpa1* mutant (Okamoto et al. 2009; Pandey et al. 2010), we found additional transporters, such as those of phosphate (PHT), lipids (LTPs) etc., to be regulated by GPA1. Even more significant is the potential role of GPA1 in nutrient responses involving nitrate and phosphate, the two most agronomically important and environmentally sensitive nutrients (López-Bucio et al. 2003). Interestingly, none of the earlier studies on GPA1 mutant transcriptomes specifically reported any nutrient transporter or nutrient response to be GPA1-regulated, even though *NRT2.1* and *PDR12* were found in their raw data (Table 3).

Nitrate response

We found many genes involved in nitrate response to be up-regulated in *gpa1-5*, such as nitrate transporter (*NRT2.1*), isocitrate dehydrogenase (*ICDH*), asparagine synthase (*ASNI*), apart from the differential regulation of certain nutrient and other stress-related gene categories such as peroxidases, kinases

and cytochrome P450-s. These are significant in the light of our earlier findings on the upregulation of nitrate reductase gene expression by cholera toxin in maize (Raghuram et al. 1999) and rice (Ali et al. 2007) and its down-regulation in the GPA1 mutant (unpublished data). In addition, our finding on the regulation of *LBD29* suggests a possible role of GPA1 in lateral root development. Interestingly, lateral root development has also been associated with hormone as well as nutrient response (Forde 2014) and thus prompts further research into the relationship between GPA1, *LBD29* and nitrate-regulation of lateral root development and N-use efficiency. Taken together, these findings on the role of GPA1 in nitrate transport/response may add significant new details to the recent finding regarding the role of a heterotrimeric G-protein gamma subunit in N-use efficiency in rice (Sun et al. 2014). Further characterization of nitrate response in the *gpa1-5* mutant is underway.

Phosphate response

Interestingly, all the three DEGs related to phosphate response seem to be downregulated in the *gpa1-5* mutant. They include *WRKY75*, a transcription factor involved in phosphate starvation response (Devaiah et al. 2007; Pant et al. 2015), *PHT1*, a phosphate transporter and *LPR1*, a gene for low phosphate root. These findings, combined with our recent data (Chakraborty et al. 2015), on the role of GCR1 in regulating multiple genes related to phosphate starvation (Lopez-Arredondo et al. 2014) highlight the potential importance of G-protein signalling in P uptake/metabolism and possibly P-use efficiency. A schematic summary of the regulation of all the DEGs related to P response/starvation/efficiency by GCR1 and/or GPA1 is shown in Fig. 7. Considering that N and P are the most important determinants of plant nutrient use efficiency in general and crop fertilizer use efficiency in particular, further research on the role of G-protein signalling in nutrient/fertilizer use efficiency merits serious attention.

At least half of the GCR1/GPA1-regulated processes are shared

The upstream events of plant G-protein signalling are not unequivocally characterized, and the existence/role of G-protein coupled receptors in plants

has been a subject of some controversy (Urano et al. 2013). We analysed the genomewide impact of a knock-out mutation in *Arabidopsis* GCR1 (Chakraborty et al. 2015), the most studied and by far the best candidate for plant GPCR (Taddese et al. 2014). By comparing those results with the present results on GPA1, we asked whether the genomewide responses of GCR1 and GPA1 have anything in common. We found that 104 genes were identically regulated in both of the mutants (Fig. 9), comprising over 26 and 30 % of the total GPA1-responsive and GCR1-responsive genes **respectively**, identified in our microarray experiments. This is a clear evidence of GCR1 and GPA1 mediating identical regulatory outcomes for the first time. Such identical regulation spanning 104 genes on a genomewide scale can only be explained either by GCR1 and GPA1 working in tandem in the classical GPCR-G-protein signalling pathway, or by convergence of their independent signalling pathways at the level of gene regulation.

While these possibilities need further experimentation, our comparison of the data from both the mutants revealed a far better overlap in terms of the processes/pathways, than in terms of the genes involved. As many as 57 processes were identically affected in both the mutants, comprising 72 % of the 79 GPA1-responsive and 57 % of the 100 GCR1-responsive processes, or nearly half (46.7 %) of all the processes affected in both the mutants. The common processes include biotic stress or defense response, and secondary metabolism, specifically flavonoid biosynthesis. We compared the flavonoid biosynthesis pathway in both the mutants and found that a few steps are commonly regulated by GCR1 and GPA1 by identical regulation of the same genes, while rest are independently regulated by them (Fig. 10). The commonality in phosphate response has already been discussed above.

Among the processes that are unique to GPA1 response and therefore not found in the GCR1 mutant, we found aging, seed and fruit development, cellular nitrogen compound metabolism etc. These may be regulated through some other GCR, or independent of any GCR, as proposed by the group of Alan Jones (Urano and Jones 2013). Similarly, among categories/processes that are unique to GCR1 response and therefore not found in the GPA1 mutant, we found cell death, RNA biosynthetic process etc. We also found that the involvement of GCR1 in secondary metabolism goes beyond flavonoid biosynthesis, unlike GPA1. It is possible that they may be regulated through some other yet-to-be

identified $G\alpha$, or independent of any $G\alpha$.

Our comparison of the DEGs from other GPA1 mutant transcriptomes (Booker et al. 2012; Delgado-Cerezo et al. 2012; Okamoto et al. 2009; Pandey et al. 2010) with our GCR1-responsive DEGs (Chakraborty et al. 2015) also revealed up to 86 common genes (Booker et al. 2012) and also contributed 10 additional genes not found in our own GPA1 dataset, indicating their consistency with our basic finding that they are co-regulated by GCR1 and GPA1. It is not surprising that our study found the highest number of common genes (104) between GPA1 and GCR1, and that most of the others common genes are subsets of our list, as both our transcriptome analyses were done parallelly under identical experimental conditions. Furthermore, we validated eight of the common genes by RT-PCR and found them to be identically regulated. These results, and the finding that the similarities are even higher in some of the broad functional categories of genes, as well as in their relative hierarchy (Table 3) lends further credence to the potential co-regulation of these processes by GCR1–GPA1. Overall, these results indicate the gross similarity in many of the broad process categories regulated by GCR1 and GPA1, not only in our own studies but also from others, strongly indicating the possibility of GCR1–GPA1 coupling in the regulation of these genes/processes. An important contribution of our study is the identification of genes for which the above predictions may be tested, using them as a starting point.

Conclusions and prospects

Overall, our results show the extensive genomewide role of GPA1 in *Arabidopsis*, in regulating at least 394 genes belonging to over 79 processes/pathways, and revealing many hitherto unknown roles of GPA1 and plant heterotrimeric G-proteins in general, well beyond those reported by signal-specific or response-specific approaches. More importantly, the identical regulation of 114 genes in the GPA1 and GCR1 mutants, constituting even higher commonality at the process level, has been revealed for the first time. In other words, GCR1 and GPA1 regulate not only some common genes, but also different genes belonging to the same pathways to achieve similar regulatory outcomes, apart from playing some totally independent roles. Many of these regulatory roles have potential agronomic significance, such as stress and nutrient response, which merit further attention. Thus, our study not only

provides the most comprehensive understanding of the organism-wide role of GPA1, but also provides compelling genetic evidence to revisit the role of GCR1 in plant signalling in general, and in heterotrimeric G-protein signalling in particular.

Materials and methods

Isolation of *gpa1-5* mutant

A T-DNA tagged mutant population of 60,480 kanamycin-resistant lines (transformed with a derivative of the T-DNA vector pD991 from Dr. Thomas Jack) of *A. thaliana* ecotype Ws-2 from the *Arabidopsis* Knockout Facility at the University of Wisconsin (Sussman et al. 2000) was screened by PCR for disruption of *GPA1* gene. The mutant *gpa1-5* was detected by DNA gel blot analysis of PCR-amplified products in DNA super-pool 30 of the Kanamycin population using a combination of GPA1-specific primer KK83 [located upstream of the ATG start codon of *GPA1* ORF] (5'-CAAGTATTTGTTTTAGCTGTGGAGCTTG-3') with the left T-DNA border specific primer JL202 (5'-CATTTTATAATAACGCTGCGGACATCTAC-3'). The PCR reagents were 1× Takara Ex-Taq polymerase buffer (Takara), 0.2 mM dNTPs, 0.24 pmol/μL gene-specific primers, 0.24 pmol/μL JL202 primer, and 0.05 unit/μL Takara Ex-Taq polymerase. PCR conditions were 96 °C for 5 min and 36 cycles of 94 °C for 15 s, 65 °C for 30 s, and 72 °C for 2 min. Sequencing of KK83–JL202 PCR products revealed a single T-DNA integration into the 8th intron of *GPA1*. The mutant was backcrossed 5 times to remove any second site mutation, if present.

Seeds corresponding to the identified super-pools and sub-pools hits were grown and DNA was extracted from leaves for genotyping and sequencing of the mutant lesions. For *gpa1-5*, primers KK83 and KK86 [located down-stream of the *GPA1* ORF stop codon] (5'-CGAGACACATTAGATTTTGAATCGCTAAG-3') were used to detect the *GPA1* wild-type copy, and primers KK83 and JL202 were used to detect the presence of the T-DNA in the *GPA1* gene. PCR conditions were as above. PCR products were separated on agarose gels, and individual segregating plants for *gpa1-5* were genotyped based on the presence or absence of wild-type and T-DNA bands.

Phenotypic characterization of the mutants

Seeds of the wild type and the mutant were surface sterilized using 70 % ethanol and washed thrice with sterile ultrapure water and stratified at 4 °C for 2 days on half-strength B₅ plates. The plates were then kept in growth chamber maintained at 22 ± 1 °C with a light intensity of 150 $\mu\text{M s}^{-1} \text{ m}^{-2}$ and a photoperiod of 16:8 (light:dark). 10 day old plantlets were then transferred to 3.5 cm pots containing 1:1 mixture of soilrite and vermiculite. The pots were watered using sub-irrigation. The plants were allowed to grow for full life cycle and various phenotypic characters were measured.

Plant material and RNA isolation

Arabidopsis thaliana G-alpha (*gpa1-5*) and the corresponding wild type, were grown on 1X B5 medium hydroponically in a growth chamber at 22 ± 1 °C with a light intensity of 150 $\mu\text{M s}^{-1} \text{ m}^{-2}$ and a photoperiod of 16:8 h of light:dark cycle. The seeds were stratified prior to inoculation at 4 °C for 2–3 days. Total RNA was isolated from 3 to 4 week old whole seedlings as described previously (Pathak and Lochab 2010). RNA samples were analyzed by Nanodrop spectrophotometer and Bioanalyzer (Agilent technologies, Santa Clara, USA) to determine the quality, quantity and suitability for microarray. The isolated RNAs were also used for validating the mutants using qPCR with gene-specific primers.

Microarray experiments and data processing

Microarray experiments were performed using Agilent 8 × 60 k *Arabidopsis* array (AMADID 037661) with independent biological duplicates both the wild type, Ws2 and *gpa1-5* mutant. Total RNA was transcribed into Cy3 labelled cRNA using Agilent Quick-Amp labelling kit as per manufacturer's instructions. Labelled cRNA was purified using RNeasy minikit (Qiagen) and the specific activity of cRNA was determined as a quality control for all the samples. They were hybridized with the microarrays using Agilent in situ hybridization kit as per manufacturer's instructions. The washed slides were scanned and the images were manually verified to ensure that they are devoid of uneven hybridization, streaks, blobs and other artifacts. Hybridization across the slide was analyzed based on the number of features that were positive and significantly above background, i.e. $g(r)$ is PosAndSignif. Overall the

microarray images were clean, had uniform intensity and with very low background noise. The data was then extracted from images by using Feature Extraction 10.7 software (Agilent Technologies).

Data analysis

The data were then normalized using the recommended 'Per Chip and Per Gene Normalization' feature of the software GeneSpring GX Version 11.5. The correlation of replicates was checked using principal component analysis and correlation coefficients were obtained. The geometric mean (geomean) fold change values are represented as log2. The average data of biological replicates was taken for final calculations. Log2fold change value of 1.0 with p value of 0.05 was taken cut-off for differential-regulation. The Benjamini Hochberg FDR procedure at a cutoff value of $p \leq 0.05$ was used for multiple testing corrections. The area-proportional Venn selections were done among the differentially regulated gene list in the *gpa1-5* and the *gcr1-5* mutants using free online software (<http://bioinforx.com/free/bxarrays/venndiagram.php>).

Functional classification of DEGs

The differentially regulated gene lists were assigned gene ontology terms according the *Arabidopsis* Information Resource (TAIR 10) (Lamesch et al. 2011). The differentially regulated gene lists were subjected to enriched GO categorization using AgriGO with default settings. Pathway analysis of the DEGs to obtain the list of changed pathways was done using plant MetGenMAP, which takes AraCyc as the background. Differentially regulated transcription factors were compared with the Plant Transcription Factor Database (plantTFDB ver 2.0) (Zhang et al. 2010). Further functional classification was also carried out using Mapman tool, where the DEGs were assigned to different biological processes (bins). This tool also takes into account the log2fold change and represents it as coloured boxes on the software generated biological process map.

Data validation using qPCR

Differentially expressed genes obtained from microarray analyses were verified by RT-qPCR using Stratagene Mx3000P (Agilent technologies). Typically, total RNA was digested by RNase-free DNase (Fermantas), re-purified, quantified

and 5 µg of RNA was used for cDNA preparation for each biological replicate using Oligo(dT) primers and RevertAid reverse transcriptase (Fermentas). The analyses were done using biological triplicates, out of which two were the same as used for microarray. Sequences for designing the primers were obtained from TAIR. PCR amplifications were performed in 20 µl reaction volume by using the BrilliantIII Ultrafast SYBR Green QPCR mastermix (Agilent Technologies) with 1.0 µl of sample cDNA and 100 n moles of each gene-specific primer. Primer efficiency was determined by serial dilution of the template and only primers that worked at 90–110 % efficiency were used for all qPCR analyses. The specificity of primer pairs was obtained by melting curve analysis of the amplicons. Actin2 (ACT2) was used as an internal control for normalization. Quantification of the relative changes in gene expression was performed by using the Pffaf1 method (Pfaffl 2001).

Acknowledgments

This work was supported in part by research grant to NR [60(0056)/02/EMRII and 38(1246)/10/EMRII] and research fellowships to NC (09/806(015)/2008-EMR1) and PS (60(0056)/02/EMRII) from the Council of Scientific and Industrial Research (CSIR), Government of India. This paper is dedicated to the co-author Priyanka Sharma who is no more.

Author contributions NC performed the phenotypic analysis of the mutant, microarray and RT-qPCR experiments, analyzed the data and wrote the initial draft of the manuscript; RRP, PS and NR conceptualized the project; KK generated the mutants; DC provided initial training for data analysis to RRP and PS; NR assisted NC in finalizing the manuscript; NR and RAH supervised the project.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary material 1 (DOCX 43 kb)

Supplementary material 2 (TIFF 2027 kb)

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